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PARTICLE CLASSIFICATION AS MARKER

The present invention relates generally to screening methods and selective identification of target substances.

Background of the invention

In the pharmaceutical area i.a. it is often necessary to test very large numbers of substances against one or more target compounds to find out if any of the tested substances possess affinity towards the target, or in some other way interacts with the target. This normally is preformed by binding each candidate compound to some matrix e.g. a particle, loading a number of the particles in one reaction vessel of some kind, and incubating with the target substance. Either the candidate compound or the target is marked with identifiable groups such as fluorescent or radioactive groups or nuclei. After the reaction is deemed to have come to completion analysis of the vessel is performed to see if the marker substance or group is present, indicating that the target has indeed reacted with the substance bound to the matrix.

Screening large numbers of substances require of course large numbers of vessels, which is laborious and tedious to handle. 10000 compounds would require 10000 individual reaction vessels to be handled.

Another possibility is to mark particles with say 10000 different ligands. A mixture is prepared comprising particles having 100 different ligands and the mixture is placed in a well of a microtiter plate. 100 such mixtures are prepared totaling 10000 different ligands and said mixtures are placed in one well each. All wells are exposed to and incubated with the target substance marked with a fluorescent moiety. If fluorescence is detected in one well one knows that a hit is present in that well. A second experiment is performed where the 100 different ligands in said well are individually placed in one well each, and the incubation and exposure is repeated. The well exhibiting fluorescence will then contain the desired substance. Thus, it is necessary to perform two sets of experiments.

Therefore, it would be desirable to have access to methods and means for rendering such screening procedures less time and space consuming, and to enable the procedure to be carried out in one step.

Summary of the Invention

Therefore the present invention seeks to provide a method of identifying one or more substances having affinity for a given target substance, that would require a smaller number of reaction vessels than what is necessary today, thereby speeding up the process of investigations substantially.

This object is achieved by the method according to the invention as defined in claim 1.

In a second aspect there is provided a set of individually distinguishable particles of a matrix material, suitable for binding candidate compounds, and usable in screening procedures.

A third aspect of the invention is the use in screening procedures, of a set of individually distinguishable particles of a matrix material, suitable for binding candidate compounds, characterized in that the fact that the particles are distinguishable is used as a marker for each said candidate compound.

In particular selecting the distinguishable property to be different particle size is very powerful in this respect.

Brief Description of the Drawings

The invention will now be described with reference to examples and to the drawings, in which

Fig. 1 is a schematic representation of a micro titer well in which a mixture of 100 different particle-ligand combination has been incubated;

Fig. 2 is another schematic representation of a micro titer well;

Fig. 3 is photograph of the contents of a microtiter well taken through a fluorescence microscope; and

Fig. 4 is a graphical output from an image analysis.

Detailed Description of Preferred Embodiments

For the purpose of this application, the term "particle class" is taken to mean particles having at least one property distinguishing them from other particles of another "class". One such property can be size (diameter), another the density. Still another kind of "particle class" is formed when a number of particles of one class having a first ligand attached, is used together with particles of the same class but having a second ligand attached thereto, but where the ratio of the number of particles having different ligands is different, e.g. 2:1.

The term "sub-class" is taken to mean particles having as common features, the features or properties of a "particle class", and having been treated in some way so as to be distinguishable from another "sub-class". A "sub-class" is e.g. formed when particles of one size are provided with one ligand, and another "sub-class" is formed when the same particles are provided with a second ligand, different from the first.

The term "ligand" shall mean one moiety attached to a particle or a group of such moieties.

The basic idea behind the invention is the insight that in a mixture of objects, wherein groups of objects in said mixture have some distinguishable and identifiable feature in common within each group, it is easy to identify each group of objects. Also it is easy to identify if there has been a change in the property of the objects in one group. If for example a mixture consists of all white golf balls, tennis balls and footballs, it is very easy by visual inspection to determine that say the golf balls have changed color to green.

This insight has been applied by the inventor in the field of screening for substances having some desired property amongst large numbers of candidate substances.

An extremely simplified example is the case where two ligands are to be examined for affinity to a target substance suitably marked, e.g. with a fluorescent marker. Each ligand would then be bound to particles with mutually different size and then the particles are mixed. The mixture is placed in well of a micro titer plate and incubated. After incubation the well is washed. If the target selectively binds to one ligand it will be an easy matter to identify which ligand has bound since one can measure the size of the fluorescing particles and thereby determine which the ligand is.

As another simplified and illustrative but slightly more complex example, let us assume that one is interested in testing four ligands for affinity to some macro-molecule, e.g. a protein. Let us also assume that each different ligand is individually bound to a particle. Furthermore we assume that the particles are of only two different classes, in this case we assume two different particle sizes. Two mixtures are prepared, each comprising two sets of particles having distinguishable size, and having different ligands bound thereto. The two mixtures are placed in one well each and incubated with the macro-molecule, appropriately marked with e.g. a fluorescent marker. Thus, we have two wells each containing the same two different particle classes, but having mutually different ligands bound to them. After incubation the wells are washed to remove non-bound substances. If the macro-molecule binds to one ligand it will be a straight forward matter to identify which one by determining which particle size and which well exhibits fluorescence. This is most easily done under a fluorescence microscope, and can be done by ocular inspection in a case like the described, where there are only few wells and few ligands present. Other methods of detection are available, e.g. so called flow cytometry in combination with Coulter-counter techniques. These methods are well known to the skilled man and need not be discussed in detail herein.

For production screening using large numbers of ligands and particles in large numbers of wells, image analysis by computer using specialized software is more feasible. An example of such commercial software is LEICA® Q 500 MC.

The skilled man will appreciate that the principle demonstrated above is applicable to larger numbers of different particle sizes and large numbers of wells, as will be explained by further examples below.

The basic principle of a first embodiment of the invention may be said to encompass identification of a ligand having reacted with a target substance, by two parameters, namely a) particle size, and b) coordinate for the particle on the micro titer plate (simply in which well it is present).

Still another property of the particles that is usable as an identifying "marker" is the density. It is relatively easy to make particles having well defined densities. If ligands are bound to such particles, mixtures of said particles can be separated by centrifugation in a density gradient, and fluorescent bands in the test tube after centrifugation can be easily determined, and the position along the tube will indicate which density fraction is a hit.

The basic principle can be further elaborated by making mixtures containing different number ratios between particles of the same size having different ligands, and providing them in the same well. Thus, if two different ligands are bound to one and the same particle size but in two fractions, and these two fractions are mixed e.g. in a ratio 1:2, of course it will be an easy matter to determine to which fraction the target has bound by the fluorescence intensity being either 1/3 or 2/3 of the maximum possible, if all particles would have fluoresced.

The particles to be used may be made of any material to which suitable ligands of interest can be attached or bound. The particles are preferably hydrophilic and built up of one or more polymers which are insoluble in water. Hydrophobic polymers that have been derivatized to become hydrophilic are included. Suitable polymers are polyhydroxy polymers, e.g. based on polysaccharides, such as agarose, dextran, cellulose, starch, pullulan, etc. and completely synthetic polymers, such as polyacrylic amide, polymethacrylic amide, poly(hydroxyalkylvinyl ethers), poly(hydroxyalkylacrylates) and polymethacrylates (e.g. polyglycidylmethacrylate), polyvinylalcohols and polymers based on styrenes and divinylbenzenes, and copolymers in which two or more of the monomers corresponding to the above-mentioned polymers are included. Polymers, which are soluble in water, may be derivatized to become insoluble, e.g. by cross-linking and by coupling to an insoluble body via adsorption or covalent binding. Hydrophilic groups can be introduced on hydrophobic polymers (e.g. on copolymers of monovinyl and divinylbenzene) by polymerization of monomers exhibiting groups which can be converted to OH, or by hydrophilization of the final polymer, e.g. by adsorption of suitable compounds, such as hydrophilic polymers.

The particles can also be based on inorganic material, such as silica. Preferred particles lack hydrolytically unstable groups, such as silane, ester and amide groups.

The particles may be porous.

The term "hydrophilic particle" in practice means that the accessible surface of the particles is hydrophilic in the sense that can be penetrated by aqueous liquids. Typically the accessible surfaces on a hydrophilic particle expose a plurality of polar groups for instance comprising oxygen and/or nitrogen atoms. Examples of such polar groups are hydroxy, amino, carboxy, ester, ether of lower alkyls (such as $(-CH_2CH_2O-)_nH$ where n is an integer).

Suitable size fractions of such particles are obtained by sieving, using standard methods well known to the skilled man. The resulting particle fractions will have some spread in terms of average diameter, but the spread can be controlled so that overlap between fractions can be adequately controlled.

Furthermore, a certain overlap in fraction size is no problem since the image analysis software that calculates average particle sizes is able to distinguish with a high degree of certainty that to which fraction the fluorescing particles belong..

Another possible material for the particles is poly-styrene. By using this material it is possible to make mono-disperse particles without overlap in fraction size.

In order to prepare suitable mixtures when the number of particles and ligands is large, so called factor design is preferably used. The theory behind this methodology is disclosed in *"Statistics for Experiments"* by Box et al, ISBN 0-471-09315-7.

The invention will now be further described by way of the following non-limiting examples.

EXAMPLES

Example 1

10 particle fractions ranging in size from fraction 1 of 10-20 μm up to fraction 10 of 100-110 μm are prepared. Each size fraction is further subdivided into 10 density fractions ranging from 1,01 up to 1,19 with increments of 0,02. Thus, in all 100 different sub classes of particles defined by both a) size and b) density is produced. To particles of each sub class a different ligand is bound to provide 100 unique combinations of ligand and particle.

10000 different ligands are to be tested. Thus, the 100 particle subclasses are used to provide 100 sets of 100 particle-ligand combinations. Each set of 100 combinations is placed in one well each of a 100-well microtiter plate. Then all wells are incubated with a target compound. The compound is suitably marked with a fluorescent moiety. In addition to being identifiable by the two properties of the particle (size and density), each ligand is identified by a coordinate, i.e. the well number.

After incubation the wells are washed to remove all target compounds that have not bound to any ligands.

Fig. 1 shows a matrix symbolizing one well in which it has been determined that the size fraction 70-80 μm exhibits fluorescence (symbolized by the particles being filled), and thus contains a subclass of particles to which the target compound has bound. Of course only 10% of the particles of this size will fluoresce, but it cannot be decide which ligand has reacted.

In order to determine which density fraction contains the target bound to the particle, the entire contents in the well is centrifuged in a density gradient, so as to yield bands corresponding to each density. The band (density 1,11) containing the fraction having target bound thereto will then fluoresce. This is symbolized by the dots representing the density fraction in question being filled.

The cross section of the two rows will identify the particular ligand that has bound to the target molecule.

Example 2

In order to double the number of possible ligands to test (or alternatively to reduce the number of wells needed for the screening), it is also possible to use the number ratio between subclasses of particles as a marker.

Thus, 10 particle fractions ranging in size from fraction 1 of 10-20 μm up to fraction 10 of 100-110 μm are prepared. Each size fraction is further subdivided into 10 density fractions ranging from 1,01 up to 1,19 with increments of 0,02. Thus, in all 100 different sub classes of particles defined by both a) size and b) density is produced. To particles of each sub class a different ligand is bound to provide 100 unique combinations of ligand and particle.

20000 different ligands are to be tested. To this end, the 100 particle subclasses are used to provide a first lot of 100 sets of 100 particle-ligand combinations, which yields 10000 combinations. Furthermore, a second lot of 100 sets of 100 particle-ligand combinations, but with 10000 other ligands than in the first lot are made. In one and the same well one set of 100 particle-ligand combinations from the first lot is combined with twice as much (total number of particles or weight of the mixture or some other measure of quantity) from the

second lot. This means that for each individual particle (having a unique size and density), there will be two possible ligands. The well is then incubated with a target molecule suitably marked.

After washing the wells to remove the non-bound target molecules, it is found that the size fraction 30-40 µm exhibits fluorescence (see Fig 2 which is a schematic representation of the contents of the well). It is also determined by simple counting or by measuring a total intensity of the fluorescence, that the number of fluorescing particles is twice as many as would have been the case if the target had reacted with particles from the first lot, and thus it must be from the second lot.

A separation by density through centrifugation is performed as in Example 1, yielding 10 bands, one of which is fluorescing (density 1,01). Again, all information needed to determine which ligand has reacted is available.

Example 3

In this example it is demonstrated that the number of possible ligands to test can be increased also by mixing the particle classes in an intelligent way.

Particles of three different particle sizes, average diameters being 15 µm, 25 µm and 55 µm respectively are used and 18 different ligands (designated A-S) are bound to these particles, thus forming 54 different combinations of ligand/particle. Thus, a combination of ligand A with a particle having the diameter 15 µm is designated A-15, ligand B bound to a 55 µm particle is designated B-55 etc.

Mixtures of these ligand-particle aggregates are prepared according to the following protocol:

Mixture No.	Composition (ligand/particle size)
1	A-15; B-25; C-55; D-15; E-25; F-55
2	G-25; H-55; I-15; K-25; L-55; M-15
3	N-55; O-15; P-25; Q-55; R-15; S-25
4	A-15; B-25; C-55; D-25; E-55; F-15
5	G-25; H-55; I-15; K-55; L-15; M-25
6	N-55; O-15; P-25; Q-15; R-25; S-55

According to this protocol, a combination can occur either in one well only, or in two different wells. For example the combination A-15 occurs in well No. 1 and 4, whereas combination D-15 occurs only in well No. 1.

These mixtures are placed in one well each (designated with the same number as the mixture numbers) of a micro titer plate, thus in six wells. The mixtures are incubated with one target substance, suitably marked with a fluorescent moiety. After completed incubation the wells are washed with water to remove all unbound target substances.

It is found that wells No. 2 and 5 exhibited fluorescence, and that the fluorescent particles have a diameter of 25 μm by image analysis. From this information it can be concluded that ligand G has bound to the target molecule, since the only combination with a particle of diameter 25 μm common to well 2 and 5 is G-25.

This being an extremely simple example, it is appreciated that the protocol of mixing can be much more sophisticated and involved, in order to distinguish one reacting species among a large number of possibilities.

In Fig. 3 a typical image viewed in a fluorescence microscope is shown. As can be clearly seen it is possible even with ocular inspection to distinguish three different particle sizes (in this case for illustrative purpose, all particles fluoresce in order to be able to see them; in a real run of course only one particle size should be visible). An image analysis by computer yields the result shown in Fig. 4, wherein also the distribution of sizes within each nominal class can be seen.

In a further development of the method, the mixtures of ligand-particle combinations are incubated with two or more target molecules. To this end the targets are suitably marked with distinguishable markers, such as fluorescent moieties exhibiting fluorescence of different wave lengths.

What is claimed is:

1. A method of identifying one or more substances having affinity for a given target, comprising:

providing a set of particle classes, each said particle class being distinguishable from another class by at least one property, those particles belonging to one and the same class having a plurality of different ligands attached to their surface, thereby forming a sub-class of particles for each ligand;

combining a plurality of sub-classes to form at least one mixture,

distributing the mixtures in separate vessels;

exposing each mixture to said substance;

washing away all target substances not having bound to any candidate substance; and

identifying to which particle sub-class(es) said target actually has(have) bound.

2. The method as claimed in claim 1, wherein one of said properties of the particle classes is the size of the particles in each class.

3. The method as claimed in claim 1 or 2, wherein one of said properties of the particle classes of the particle classes is the density of the particles in each class.

4. The method as claimed in claim 1, 2 or 3 wherein the mixtures are formed by mixing two particle classes in a ratio such that the difference in number of one class with respect to the other is detectable in the identifying step.

5. The method as claimed in any preceding claim, wherein the target substance is marked so as to be detectable.

6. The method as claimed in claim 5, wherein the marking is by attaching a moiety selected from the group consisting of a fluorescent moiety, a radioactive moiety, a colored moiety,.

7. The method as claimed in claim 5, wherein the target substance reacts with the ligand to which it binds to provide a detectable effect, such as fluorescence or color.

8. The method as claimed in any preceding claim, wherein the identification is performed by ocular inspection under microscope.

9. The method as claimed any preceding claim, wherein the identification is performed by image analysis with a computer.

10. The method as claimed in any preceding claim, wherein said target comprises one or more substances.

11. A library of ligands attached to one particle class each, each said particle class being distinguishable from another class by at least one property, those particles belonging to one and the same class having a plurality of different ligands attached to their surface, thereby forming a sub-class of particles for each ligand.

12. The ligand library as claimed in claim 11, wherein one of said properties of the particle classes is the size, suitably the diameter of the particles.

13. The ligand library as claimed in claim 11 or 12, wherein one of said properties of the particle classes is the density of the particle.

14. The use of a ligand library as claimed in claim 11 for screening purposes.

ABSTRACT:

The invention relates to a method of identifying one or more substances having affinity for a given target, comprising providing a set of particle classes, each said particle class being distinguishable from another class by at least one property. The particles belonging to one and the same class have a plurality of different ligands attached to their surface, thereby forming a sub-class of particles for each ligand. A plurality of sub-classes is combined to form at least one mixture.The mixtures are distributed in separate vessels and exposed to said substance. All target substances not having bound to any candidate substance are washed away, and it is detemined to which particle sub-class(es) said target substance actually has(have) bound.

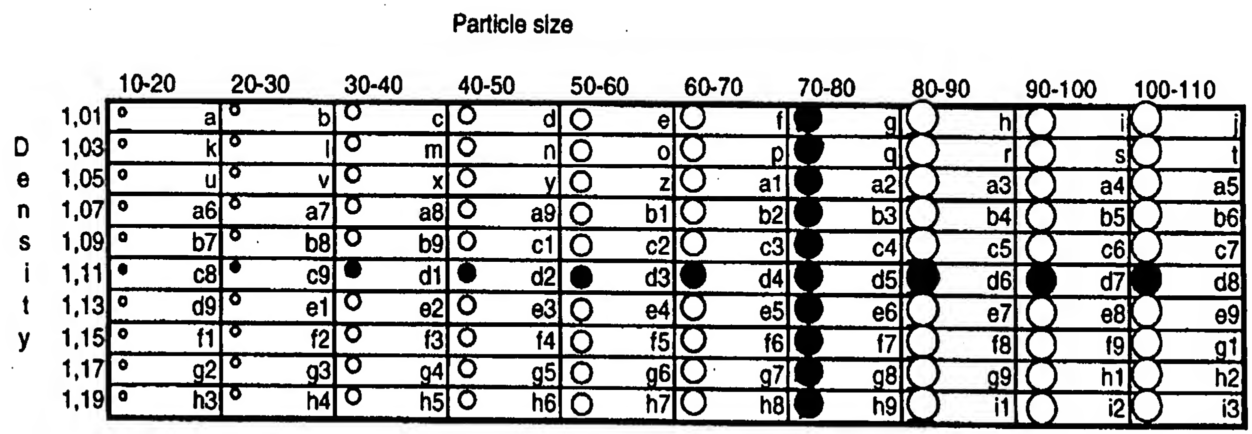
Particle size
10-20 20-30 30-40 40-50 50-60 60-70 70-80 80-90 90-100 100-110
Density
1,01 a b c d e f g h i j
1,03 k l m n o p q r s t
1,05 u v x y z a1 a2 a3 a4 a5
1,07 a6 a7 a8 a9 b1 b2 b3 b4 b5 b6
1,09 b7 b8 b9 c1 c2 c3 c4 c5 c6 c7
1,11 c8 c9 d1 d2 d3 d4 d5 d6 d7 d8
1,13 d9 e1 e2 e3 e4 e5 e6 e7 e8 e9
1,15 f1 f2 f3 f4 f5 f6 f7 f8 f9 g1
1,17 g2 g3 g4 g5 g6 g7 g8 g9 h1 h2
1,19 h3 h4 h5 h6 h7 h8 h9 i1 i2 i3

Fig. 1

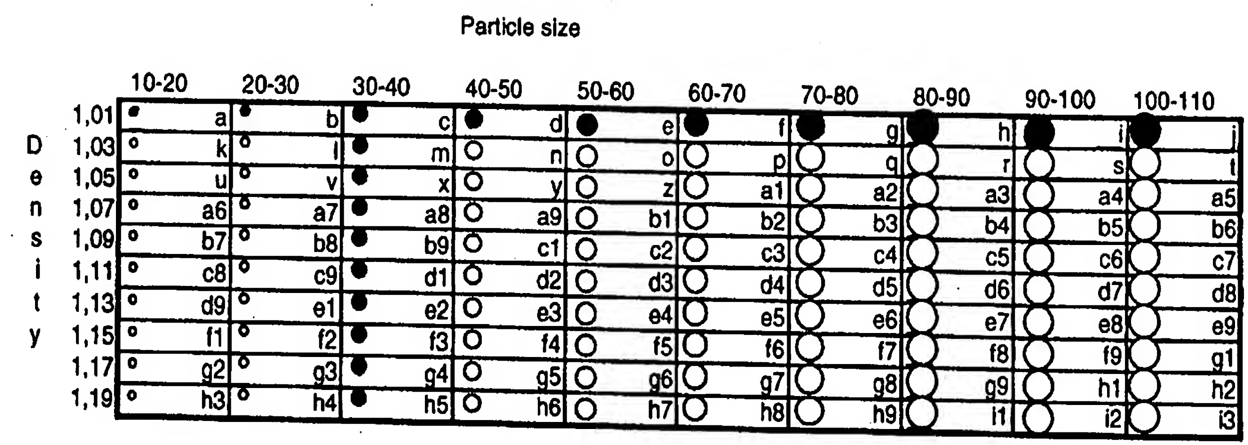
Particle size
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
100-110
Density
1,01
1,03
1,05
1,07
1,09
1,11
1,13
1,15
1,17
1,19
a b c d e f g h i j
k l m n o p q r s t
u v x y z a1 a2 a3 a4 a5
a6 a7 a8 a9 b1 b2 b3 b4 b5 b6
b7 b8 b9 c1 c2 c3 c4 c5 c6 c7
c8 c9 d1 d2 d3 d4 d5 d6 d7 d8
d9 e1 e2 e3 e4 e5 e6 e7 e8 e9
f1 f2 f3 f4 f5 f6 f7 f8 f9 g1
g2 g3 g4 g5 g6 g7 g8 g9 h1 h2
h3 h4 h5 h6 h7 h8 h9 i1 i2 i3

Fig. 2

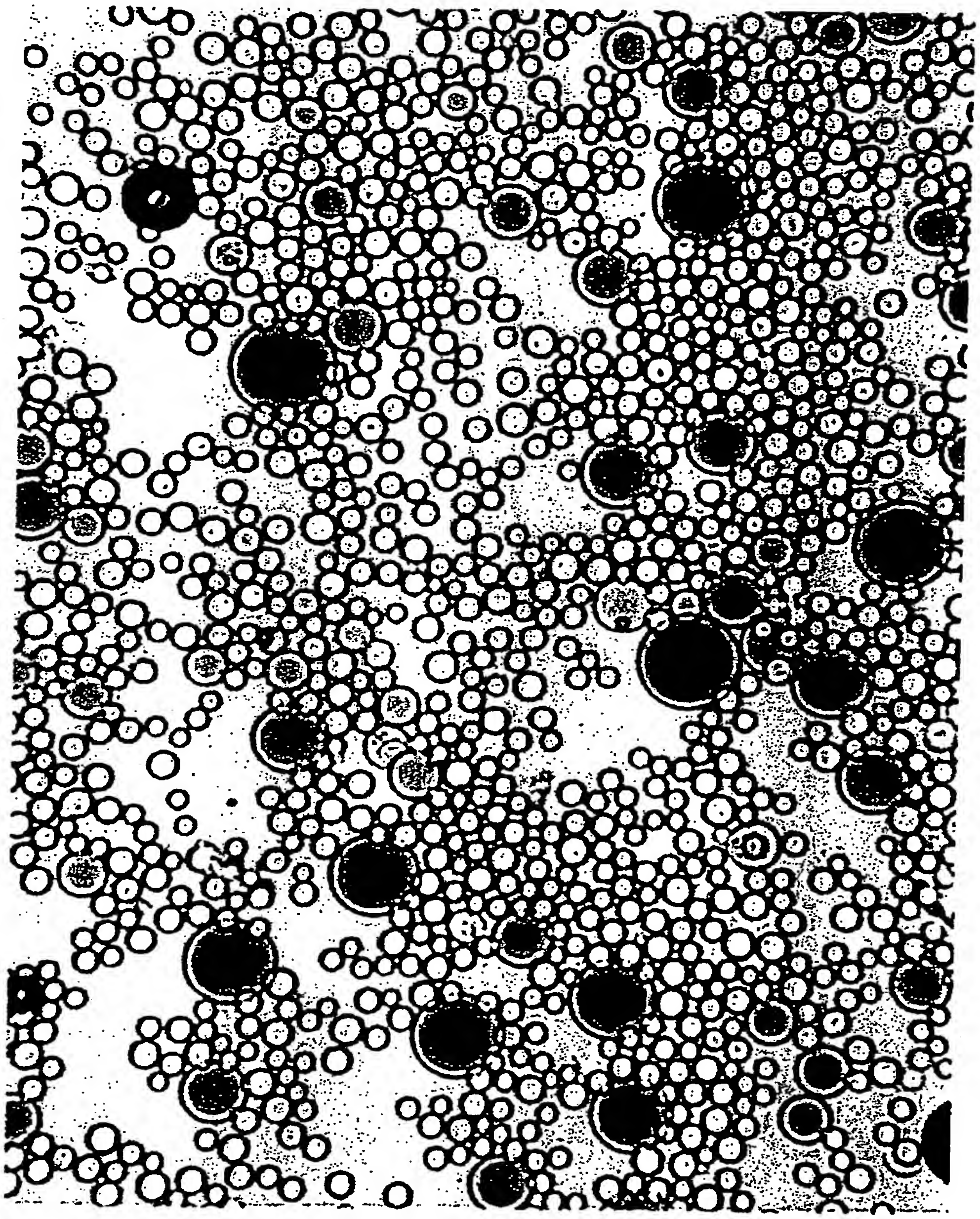

Fig. 3

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Size as a marker

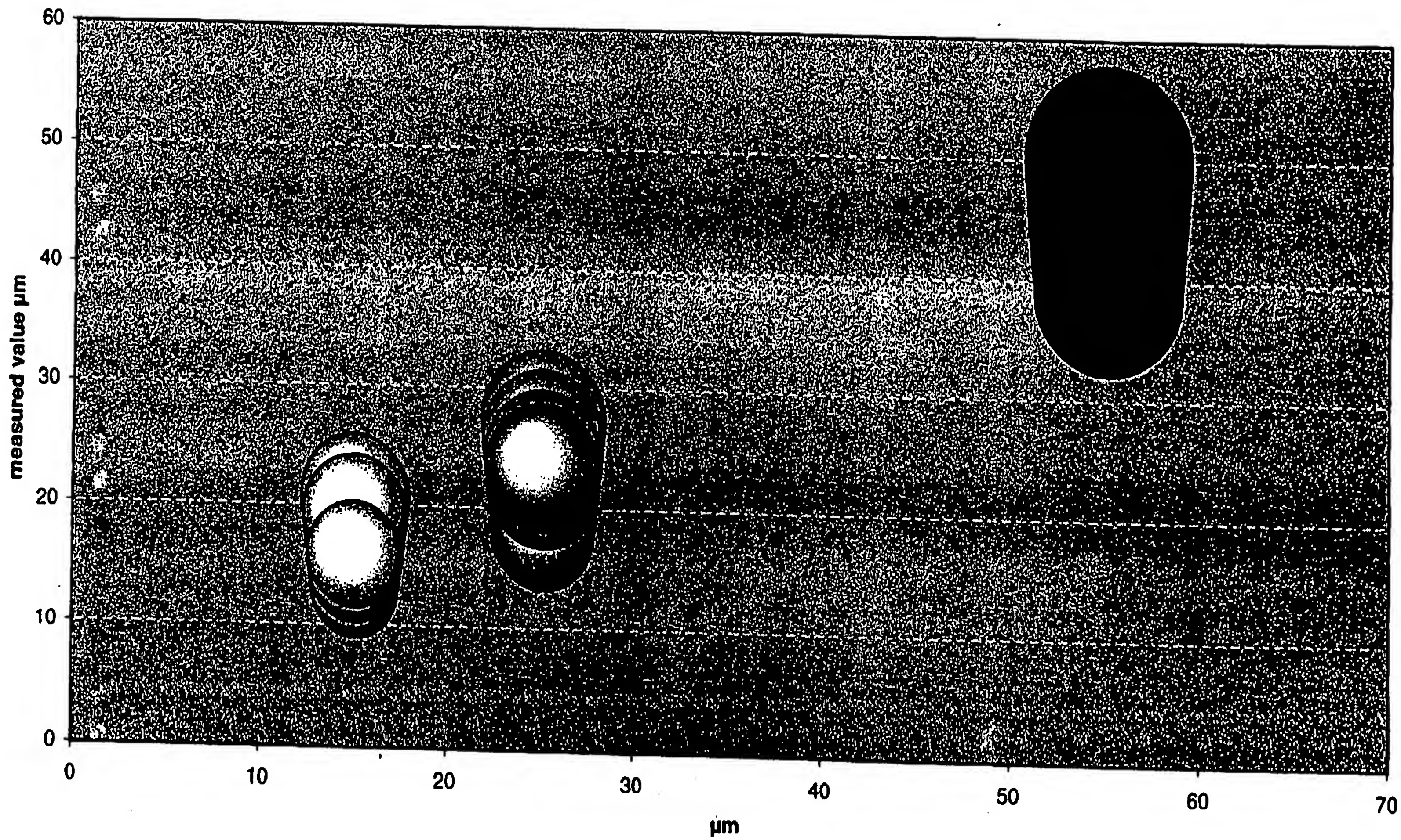

Fig. 4

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